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Distinct Role of Long 3′ UTR BDNF mRNA in Spine Morphology and Synaptic Plasticity in Hippocampal Neurons

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Supplemental Experimental Procedures

Culture and transfection of primary neurons

Hippocampal and cortical neurons were isolated and cultured according to a previously described procedure (Sala et al., 2000). Hippocampi or cortices were dissected from E18.5 rat embryos or mouse pups at age of P0-P2. Isolated brain tissues were digested with 0.025% trypsin in 1X Hanks' balanced salt solution (HBSS) at 37 °C for 15 minutes. Dissociated cells were plated on 15-mm-diameter coverslips coated with poly-D-lysine (37.5 μg/ml) and laminin (2.5 μg/ml) in Neurobasal medium containing 2% B27 supplement, 500 μM glutamine, and 12.5 μM glutamate. At 7 days *in vitro* (DIV) one half of the medium was replaced with fresh medium without glutamate, and Ara-C (10 μM) was then added. For transfection, 1 μl of lipofectamine 2000 or lipofectamine LTX and 1 μg of DNA were added to 25 μl of Neurobasal medium individually and incubated at room temperature for 5 minutes. The two parts were then mixed and incubated at room temperature for another 25 minutes before adding onto each coverslip containing cultured hippocampal neurons. Culture medium was replaced with conditioned medium (fresh medium mixed with 4-day-old culture medium at 1:1) the next morning.

Isolation of dendritic fragments and cell bodies from cortical cultures

Three week-old high-density cultures of rat cortical neurons were treated with papain (0.1 mg/ml) for 5 minutes at room temperature. The cultures were rinsed 3 times with PBS and covered with 3 ml/dish of a buffer containing 10 mM Tris-HCl (pH 7.4), 0.5 mM EGTA and 0.35 M sucrose, and passed through a wide bore pipette to break down the network. The fractions were pooled and mixed 1:1 with a 0.8 M sucrose solution prepared in the same buffer and spun at 1,500 x g for 10 min. The pellet (P1) contains cell bodies and probably large dendritic fragments. The supernatant was recovered and centrifuged at 28,000 x g for 10 min to produce a pellet (P2) which was free of somatic contamination and enriched in pre- and post-synaptic structures as evidenced by electron microscopy. The enrichment of dendritic components in P2 was also assessed by immunoblotting analysis. As shown in Fig. S2A, synaptic markers synaptophysin and GluR5 receptor were enriched in P2, while nuclear protein NeuN was only detected in P1. Glial fibrillary acidic protein (GFAP), a glial marker, was also absent in P2. In addition, RT-PCR analyses indicated the presence of dendritic mRNAs in P2, including MAP2 and BC1, whereas the γ-actin and GFAP mRNAs were absent in P2 (Fig. S2B). The primers used in RT-PCR analysis of BDNF mRNAs were 5'-tggcctaacagtgtttgcag-3' and 5'-ggatttgagtgtggttctcc-3' for the long BDNF 3'UTR, and 5'-tggctgacacttttgagcac-3' and 5'-ccagccaattctctttttgc-3' for the BDNF coding region. Approximately 5% and 100% of total RNA isolated from the dendrite fraction and the soma fraction were reverse transcribed, respectively, followed by semi-quantitative PCR

using ^{32}P-labeled primers specific for the long 3'UTR or the coding region. The ^{32}P radioactivity was determined based on phosphorimager reading.

Brain sections

Mice were anaesthetized with avertin and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde sequentially, and their brains were extracted from the skull. For immunohistochemistry, the brains were postfixed in 4% paraformaldehyde overnight and transferred to 30% sucrose in PBS. For in situ hybridization, the brains were soaked in 4% paraformaldehyde-20% sucrose-PBS for three days at 4 °C. The brains were sectioned at 40 μm with a sliding microtome. Sections were collected into a 12-well tissue culture plate containing PBS and stored at 4 °C.

In situ hybridization

To generate DIG-labeled antisense and sense riboprobes, mouse cDNA sequences for the BDNF coding region (GenBank accession number NM_001048139, nucleotides 521-1270), the long BDNF 3'UTR (GenBank accession number NM_001048139, nucleotides 2207-4161), CaMKIIα (GenBank accession number NM_177407, nucleotides 111-1619), and β-tubulin (GenBank accession number NM_023279, nucleotides 231-1203) were amplified by PCR and cloned into the pBluscript II KS (-) plasmid (Stratagene, Cedar Creek, TX, USA). The selection of the CaMKIIα and β-tubulin sequences was based on a previous report (Muddashetty et al., 2007). Antisense and sense RNA probes were synthesized from linearized plasmids by using T3 and T7 RNA polymerases and DIG-labeled nucleotides. After synthesis, riboprobes were fragmented to ~0.1 kb by alkaline hydrolysis, and used for FISH analyses of both brain tissues and cultured neurons.

In situ hybridization of brain sections was performed according to a protocol adapted from previously described procedures (Muddashetty et al., 2007; Tongiorgi et al., 1998; Xu et al., 2003). All steps were done at room temperature unless indicated otherwise, with multiple washes in between. Free-floating brain sections were washed three times (5 min each) in PBS plus 0.1% Tween 20 (PBST) and then once in 100 mM Tris-Cl pH 7.5 for 5 min. The sections were incubated with 1% sodium borohydride in 100 mM Tris-Cl pH 7.5 for 10 min to enhance tissue permeability, digested with 10-30 μg/ml proteinase K in PBST for 20 min, and fixed with 4% paraformaldehyde in PBS for 5 min. The sections were incubated with a pre-hybridization mixture (40 mM Tris-Cl, pH 7.5, 50% formamide, 0.5 μg/ml yeast tRNA, 0.5 μg/ml denatured salmon sperm DNA, 300 mM NaCl, 1x Denhart's, and 1 mM EDTA) for 2 hours at 55 °C and then with the hybridization mixture (pre-hybridization mixture plus 10% dextran sulfate and 500 ng/ml riboprobe) for 16 hours at 55 °C. After hybridization, the sections were washed three times with 2X SSC (sodium chloride-sodium citrate buffer), treated with 50 μg/ml RNase A for 1 hour at 37 °C, and sequentially washed with 2X SSC, 1X SSC, 0.5X SSC and 0.1X SSC for 5 min each, followed by one high-stringency wash in 0.1X SSC for 1 hour at 65 °C. Endogenous peroxidase activities of the brain sections were quenched by incubation with 3% H_2O_2 and 10% methanol in PBST for 20 min. The sections were incubated with Roche's blocking buffer (Roche Applied Science, Indianapolis, IN, USA) for 1 hour and then anti-DIG-POD Fab fragments (Roche Applied Science, 1:1000 dilution) in the blocking buffer overnight. After four 10-min washes in TNT buffer (100 mM Tris-Cl, pH7.5, 150 mM NaCl, and 0.05% Tween 20), in situ signals were amplified with the TSA Plus Fluorescein System (PerkinElmer, Waltham, MA, USA) according to the manufacture's instructions. The sections were mounted onto slides and

examined under a confocal microscope. In situ hybridization images from an antisense probe and its sense control probe were taken with the same settings. A series of 10 µm-wide rectangles were drawn in the stratum radiatum to measure the average intensity of in situ hybridization signals at a 10-µm step away from the stratum pyramidale by using NIH image software. The sense in situ hybridization signal in the stratum radiatum was considered as the background.

In situ hybridization of cultured neurons was performed as described (Marz et al., 1998) with modifications. Cells were fixed for 30 min in 4% paraformaldehyde, washed in PBST, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and acetylated for 10 min with 0.1 M triethanolamine hydrochloride/0.25% acetic anhydride (pH 8.0). Pre-hybridization and hybridization mixtures were the same as above. Probe concentrations were 500 ng/ml for BDNF mRNA and 100 ng/ml for GFP mRNA. After overnight hybridization, cells were treated with 25 µg/ml RNase A at 37℃ for 20 min and washed twice at 65℃ in 0.1X SSC for 30 min. Visualization of hybridized probes was performed as described for free-floating brain sections. Fluorescent MAP2 immunocytochemistry was performed as described below after the hybridization step was completed. In situ hybridization signals and MAP2 immunoreactivity were examined under a confocal microscope. The total in situ hybridization signals in a cell body and each dendritic segment were quantified by using the Image J software. After the hybridization and wash steps, green fluorescence from GFP in transfected neurons was not detectable.

Generation of GFP-BDNF 3'UTR constructs

The genomic sequences encoding BDNF 3'UTRs A, B and AB were obtained by PCR using mouse genomic DNA as templates. The primers used for the three constructs were 5'-gcggccgctggatttatgttgtatagattatattgaga-3' (forward) and 5'- cttaagcagagtactaacaagaacgaaagatac-3' (reverse) for 3'UTR A, 5'-gcggccgcttggaaccaaaacattccgtttac-3' (forward) and 5'-cttaaggatggtcttaaagcaaggaa-3' (reverse) for 3'UTR B, and the forward primer of 3'UTR A and the reverse primer of 3'UTR B for 3'UTR AB. The forward and reverse primers contain NotI and AflII restriction sites, respectively. To make GFP fusion constructs, we cloned the EGFP coding sequence from the pEGFPN1 vector (BD Bioscience, San Jose, CA) into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) at the NheI and NotI sites to generate the GFP-BGH construct. The PCR products for the three BDNF 3'UTRs were digested with NotI and AflII restriction enzymes and subcloned into the GFP-BGH construct at the same sites to generate GFP-A, GFP-B, and GFP-AB constructs. The three plasmids containing BDNF 3'UTRs were sequenced to make sure that no mutations were introduced by PCR.

Reporter of local protein synthesis

The human synapsin (hSYN) promoter was amplified from the lentiviral FSW plasmid, introducing a 5' BglII site and a 3' XhoI site, allowing insertion between these sites into pCDNA3.1(-) A (Invitrogen, Carlsbad, CA), swapping out the CMV promoter and generating phSYN-cDNA3.1. The Src tyrosine kinase myristoylation sequence, atggggagtagcaagagcaagcctaag, encoding the amino acids MGSSKSKPK, was added to the 5'-end of a PCR-amplified EGFP insert from plasmid pd1EGFP-N1, which was cloned into phSYN-cDNA3.1 between the BamHI and NotI sites, generating phSYN-myr-d1GFP. The mouse BDNF 3'UTR sequences A and A*B, where the polyadenylation signal AATAAA for the first polyadenylation site was changed to TTTTTT, were cloned into phSYN-myr-d1GFP between the NotI and AflII sites, generating phSYN-myr-d1GFP-A (myr-d1GFP-A) and phSYN-myr-

d1GFP-A*B (myr-d1GFP-A*B). These two constructs were transfected into cultured rat hippocampal neurons at 13 DIV. One day after transfection, the cultured neurons were treated with 1 μM TTX or vehicle for 6 hours and then fixed. The longest dendrites of transfected neurons were analyzed by quantifying the fluorescent intensity of a line drawn through the center of the dendrite, and mean intensity values for each condition were calculated using 50 μm bins.

Northern blots and Western blots

The probes for Northern hybridization were as follows: *Bdnf* coding region (GenBank accession number NM_001048139, nucleotides 521-1270), *Bdnf* exon 2 (GeneBank accession number AY057907, nucleotides 6998-7201), *Bdnf* exon 4 (GeneBank accession number AY057907, nucleotides 23540-23878) and *Bdnf* exon 6 (GeneBank accession number AY057907, nucleotides 25573-25725). The probes were labeled by using the Rediprime II DNA labeling system (Amersham Bioscience, Piscataway, NJ). Antibodies to BDNF (1:1000) and α-tubulin (1:5000) for western blots were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma-Aldrich (Saint Louis, MO), respectively.

Immunohistochemistry and immunocytochemistry

For immunohistochemistry, brain sections were rinsed once with Tris buffered saline (TBS: 10 mM Tris–HCl, 150 mM sodium chloride, pH 7.5) and incubated with 10% methanol-3% hydrogen peroxide in TBS to quench endogenous peroxidase. After incubating with a blocking buffer (0.4% Triton X-100, 2.5% bovine serum albumin, and 10% horse serum in TBS) to block nonspecific interactions, the sections were incubated with a primary antibody diluted in the blocking buffer overnight at room temperature. After four 30-min washes in the blocking buffer, the sections were incubated with an appropriate biotinylated secondary antibody followed by avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA) according to the instructions of the manufacturer. Sections were developed in 0.05% 3-3′-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in 0.1 M Tris–HCl (pH 7.5), mounted onto slides, dehydrated, and coverslipped with DPX. For immunocytochemistry, cultured neurons were fixed with 4% paraformaldehyde, blocked with the blocking buffer, and incubated with primary antibodies diluted in the blocking buffer overnight at room temperature. After washes, the samples were incubated with approximate Texas Red- or Cy5- and FITC-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA and Jackson Immunochemicals, West Grove, PA, USA) at 1:200 dilution, washed, mounted with Fluorescent Mounting Medium (DakoCytomation), and analyzed with a confocal microscope. Rabbit polyclonal antibody to BDNF was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used for immunohistochemistry at 1:2000 dilution and immunocytochemistry at 1:500 dilution. The mouse monoclonal antibody to MAP2 (1:500 dilution) were purchased from Chemicon International (Temecula, CA, USA).

BDNF secretion

Hippocampal neuronal cultures were plated at a density of 5 x 10^5 cells per well in a 12-well plate. On 10 DIV cells were washed sequentially as following: once with a washing medium (Neurobasal medium supplemented with B27, 500 μM glutamine, and 100 μg/ml bovine serum albumin), once with the washing medium containing 50 μg/ml protamine sulphate to block non-specific protein binding, and twice with the washing medium. For measurement of constitutive BDNF secretion, the washed cells were incubated with Neurobasal medium supplemented with

0.1 μg/ml bovine serum albumin, 15 μM tetrodotoxin (TTX), 20 μM D-2-amino-5-phosphonovaleric acid (D-APV), and 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) for 16 hours. For measurement of regulated BDNF secretion, the washed cells were treated with Neurobasal medium supplemented with 0.1 μg/ml bovine serum albumin and 50 mM KCl for 15 min. Media were collected and centrifuged at 13,000 rpm for 15 min. Supernatants were concentrated using Microcon centrifugal filter devices (YM-10, Millipore). The amount of BDNF in each medium sample was determined using the BDNF E_{max} ImmunoAssay System (Promega, Madison, WI) according to the manufacturer's instructions.

Golgi impregnation

Golgi impregnation of the whole brain was performed by using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Ellicott City, MD). We used Neurolucida software (MicroBrightField, Williston, VT) to trace dendrites. To measure spine density, we took images of distal dendrites with similar diameters in the stratum radiatum, enlarged the images with Photoshop software (Adobe Systems, San Jose, CA), and counted spines on printouts. All small protrusions on dendrites were considered as spines. Each printout was labeled with an animal identification number, so that spine density measurements could be done blind to genotypes. Spine density for each animal was obtained from many distal dendrites with a total length of 1000-2000 μm. Dendritic images from each mouse were randomly used for measurement of spine size. The densely stained area at the tip of a spine was considered as a spine head. Once a dendritic segment was selected, diameters of all spine heads on the dendritic segment were measured. We measured ~120 spines for each animal.

Electrophysiological recording

Transverse hippocampal slices (400 μm) were prepared from $Bdnf^{klox/klox}$ and age-matched wild-type mice (8-10 weeks old). Slices were maintained in an interface chamber exposed to a humidified atmosphere of 95% O_2 and 5% CO_2. After a minimum recovery period of at least two hours, extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded using an Axoclamp-2B amplifier (Axon Instruments) with an artificial cerebral spinal fluid [ACSF containing (in mM): NaCl 124, KCl 3.0, $CaCl_2$ 2.5, $MgCl_2$ 1.5, $NaHCO_3$ 26, KH_2PO_4 1.25, glucose 10, ascorbic acid 2, pH 7.4] filled glass microelectrode (1-3 MΩ) positioned in the stratum radiatum area of CA1. Tetanic stimulation was applied after stable baseline was established for at least 30 min. Stimulus intensity was adjusted to evoke fEPSPs approximately 40% of the maximum. LTP was induced by two trains of high frequency stimulation (100 Hz, 1 s, separated by 20 s) or by weak theta burst stimulation consisting of 3 bursts of 4 pulses at 100 Hz.

Whole cell recordings were made from visualized CA1 pyramidal neurons of hippocampal slices. Recording electrodes (5-7 MΩ) were filled with intracellular solution containing (mM): 144 K-gluconate, 4.5 NaCl, 2 MgATP, 0.5 HEPES, 2 Mg_2-ATP, 0.5 Na_3-GTP, 0.5 EGTA adjusted to 310 mOsm and pH 7.25. Access resistance was monitored throughout experiments and ranged from 10-25 MΩ. Data was discarded when access resistance changed by > 20% during an experiment. Action potential trains were recorded in current clamp mode in response to a series of current steps ranging from 0 to 150 pA in the presence of DNQX, APV and bicuculline methiodide. Depolarizing steps were evoked at a rate of 0.05 Hz at an initial membrane potential of -60 mV. The antagonists, APV (Sigma), DNQX (Sigma) and bicuculline methiodide (Sigma) were initially prepared as concentrated stock solutions and were diluted with ACSF to the specified concentrations prior to each experiment.

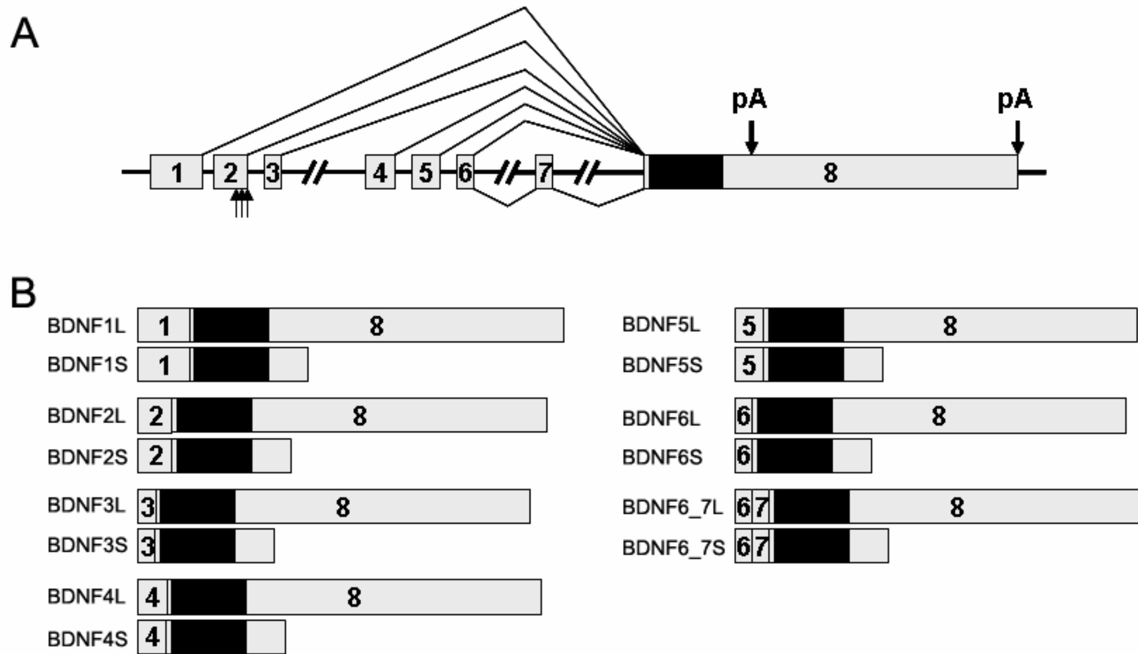

Figure S1. BDNF mRNA variants
(**A**) Diagram of the mouse *Bdnf* gene (based on Liu et al., 2006) depicting 8 exons and two alternative polyadenylation (pA) sites in exon 8 (arrows above exon 8). Curved lines linking boxes (exons) indicate alternative splicing from the first six exons to exon 8. Filled box in exon 8 represents the BDNF coding sequence. All transcripts in theory could be polyadenylated at either pA site to give rise to two mRNA species: one with a short 3'UTR and the other with a long 3'UTR.
(**B**) Diagram of 14 BDNF transcript variants produced from 6 distinct promoters preceding each of the first 6 exons. The number of variants is further increased by three alternative splice-donor sites in exon 2 (arrows underneath exon 2 in panel A).

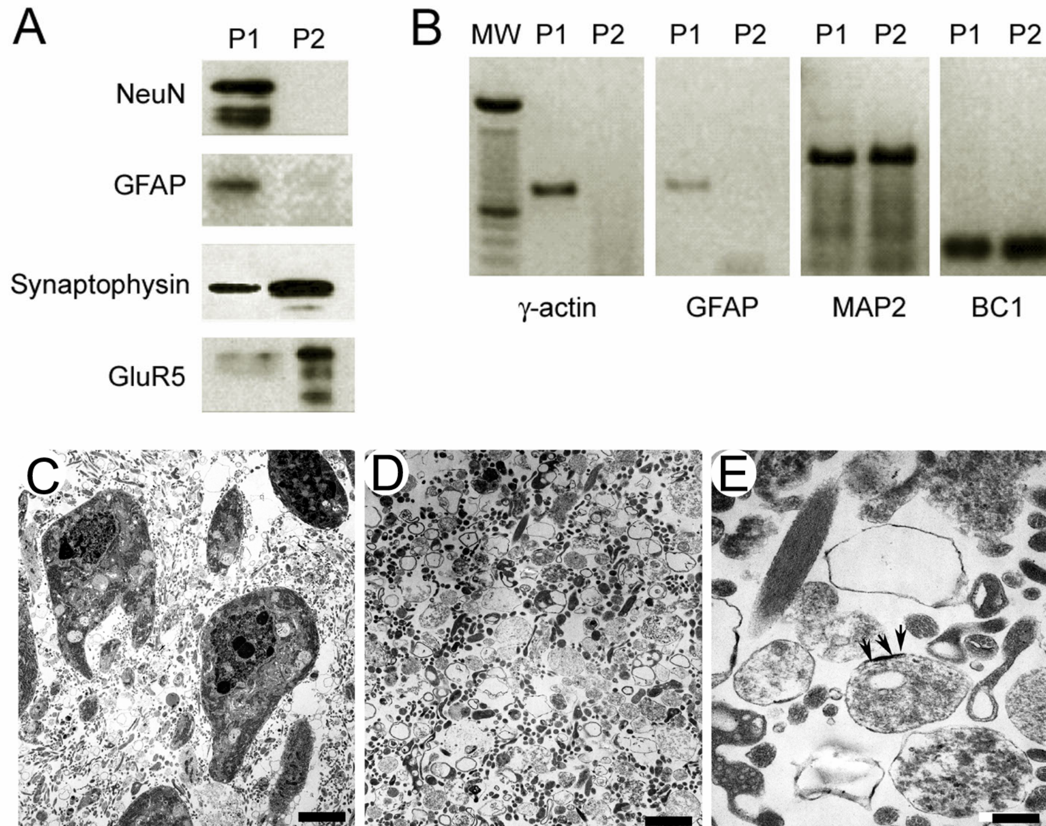

Figure S2. Isolation of synaptic components from cultured cortical neurons

(**A**) Western blots revealed that the dendrite fraction (P2) was free of the nuclear protein NeuN and the glial marker GFAP but contained synaptic markers synaptophysin and GluR5.

(**B**) RT-PCR analyses indicated that γ-actin and GFAP mRNAs were only present in the soma fraction (P1), whereas MAP2 and BC1 mRNAs were present in both P1 and P2 fractions.

(C-E) Electron microscopy of the P1/P2 fractions. The P1 fraction is highly enriched in cell bodies and mixed with neurite fragments (C). In contrast, cell bodies were absent in the P2 fraction that was enriched in pre- and post-synaptic elements (D). A higher magnification of the P2 fraction is shown in panel E. Arrows point to a post-synaptic density. Bars represent 4 μm (C, D) and 0.33 μm (E).

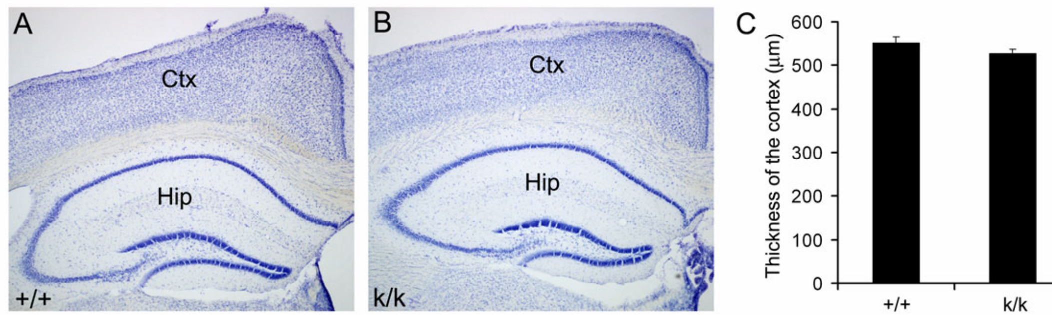

Figure S3. Normal cytoarchitecture of the cerebral cortex and the hippocampus in *Bdnf* $^{klox/klox}$ mice

(**A**, **B**) Representative images of Nissl-stained coronal sections showing comparable cytoarchitecture of the cerebral cortex (Ctx) and the hippocampus (Hip) between WT (+/+) and *Bdnf*$^{klox/klox}$ mice (k/k) mice.

(**C**) Similar cortical thickness in WT and *Bdnf*$^{klox/klox}$ mice. Thickness of the lateral parietal association cortex was obtained from coronal sections at approximate Bregma -1.9 mm (n=4 mice for each genotype, p=0.16).

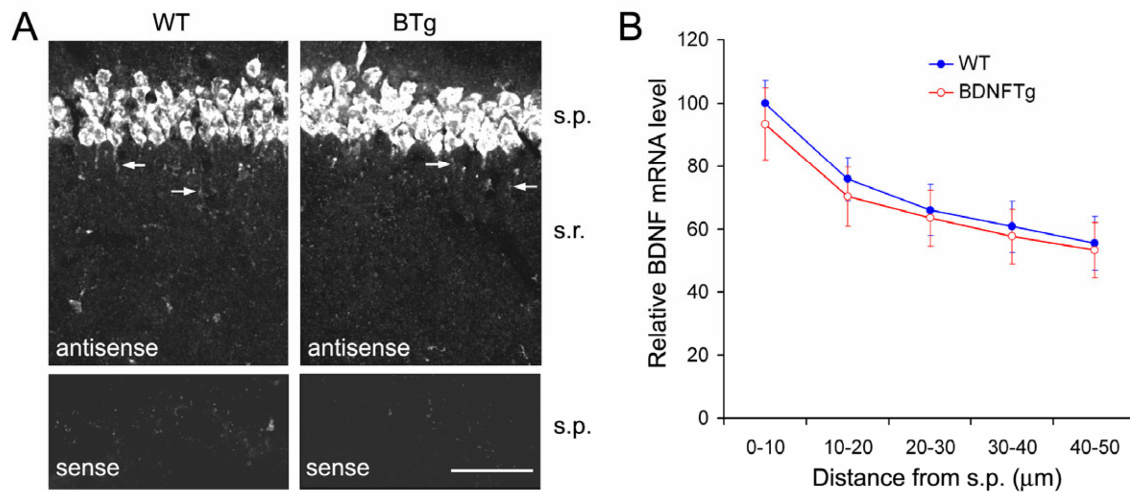

Figure S4. Similar levels of dendritic BDNF mRNA in the CA1 area of WT and BTg mice

(**A**) FISH with antisense riboprobes complementary to the BDNF coding region revealing similar levels of BDNF mRNA in the CA1 stratum radiatum of WT and BTg mice. Arrows indicate representative dendrites containing BDNF mRNA. Also note many small puncta containing BDNF mRNA in the stratum radiatum. As expected, levels of BDNF mRNA in cell bodies were elevated in BTg mice. Sense riboprobes derived from the same cDNA did not produce significant FISH signals in the stratum pyramidale. Scale bar, 50 μm.

(**B**) Quantification of BDNF mRNA in situ hybridization signals in the stratum radiatum of WT and BTg mice. The data were obtained from 4 pairs of mice (3 brain slices per mouse). No significant differences in levels of dendritic BDNF mRNA were detected between WT and BTg mice.

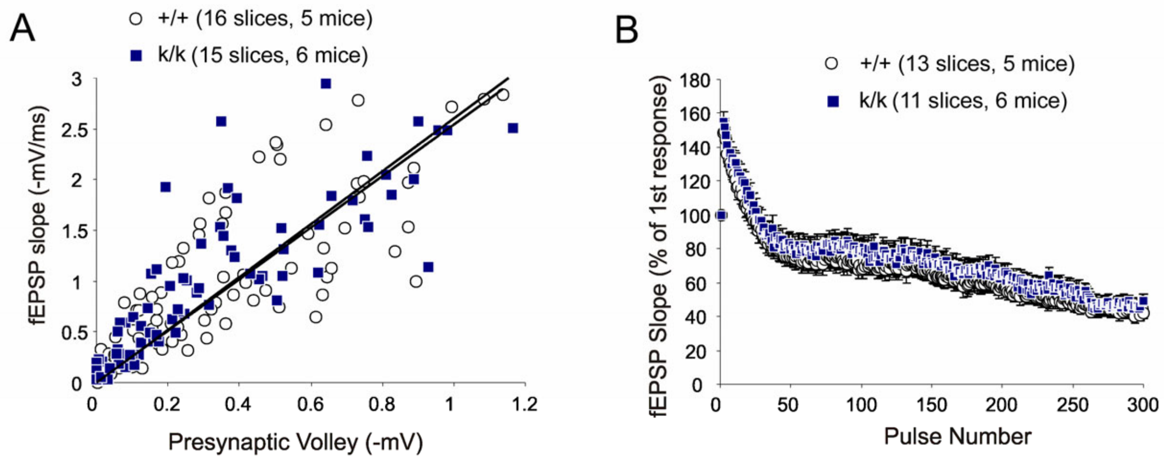

Figure S5. Schaffer collateral-CA1 synaptic responses to repetitive stimulation in *Bdnf* $^{klox/klox}$ mice

(**A**) Comparable input-output curves between WT and *Bdnf*$^{klox/klox}$ mice. fEPSP slopes plotted against their corresponding presynaptic fiber volley amplitudes showed no significant differences between *Bdnf*$^{klox/klox}$ and age-matched WT mice.

(**B**) Normal synaptic responses to prolonged low frequency stimulation (14 Hz, 300 stimuli) in the hippocampal CA1 of *Bdnf*$^{klox/klox}$ mice. Records were performed in the presence of an NMDAR antagonist, APV.

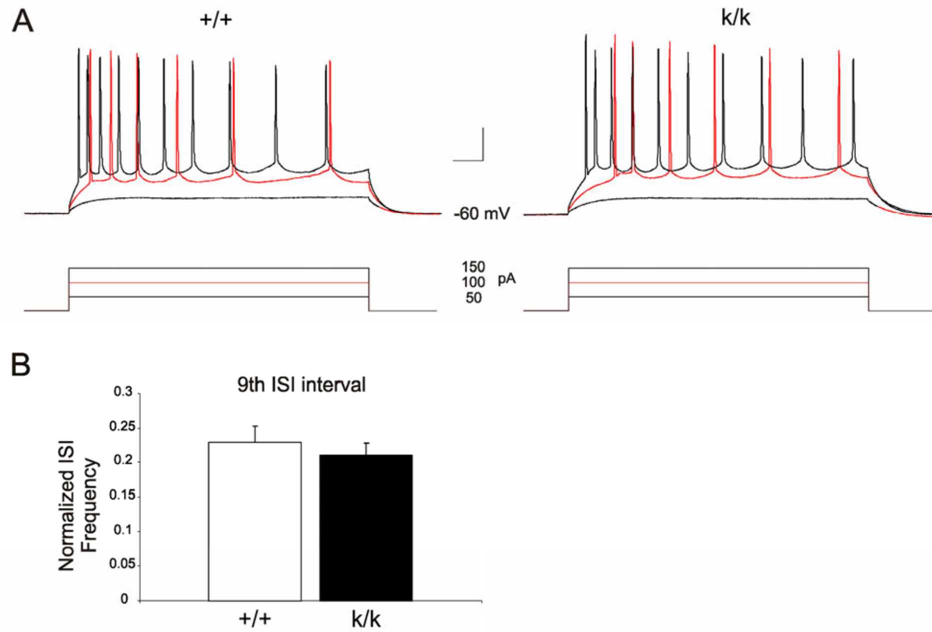

Figure S6. Normal excitability of *Bdnf*$^{klox/klox}$ CA1 pyramidal neurons

(**A**) Representative whole cell current clamp traces of action potential trains evoked by a sequence of depolarizing currents in WT and *Bdnf*$^{klox/klox}$ mice. Voltage responses (upper trace) to depolarizing current pulses (50 pA increments, lower trace), while synaptic transmission was pharmacologically blocked.

(**B**) Normal spike frequency adaptation in *Bdnf*$^{klox/klox}$ as compared to WT mice. The adaptation ratio was calculated by normalizing the prolonged 9th interspike interval (ISI) to the relatively short 1st ISI.

Table S1. Physiological properties of CA1 pyramidal neurons in WT and *Bdnf*$^{klox/klox}$ mice

Genotype	+/+	-/-
Vm (mV)	-65 ± 1	-64 ± 1
Vt (mV)	-37 ± 1	-39 ± 1
AP amp (mV)	73 ± 4	74 ± 3
AP ½ width (msec)	1.8 ± 0.1	1.9 ± 0.1

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